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ABSTRACT

Cell-free preparations from peas are described that can mediate ethylene evolution in both an enzymatic and nonenzymatic manner.

CELL-FREE ETHYLENE EVOLUTION FROM ETIOLATED PEA SEEDLINGS

Early reports of ethylene evolution from cell-free preparations have been criticized by Burg and Burg.[1] In addition, Spencer et al[2-5] and Gibson[6] have reported on cell-free ethylene evolution. Some of this work has been criticized by Meigh.[7] The unusual property of these preparations (to evolve ethylene after exposure to temperatures greater than 90°C) compromises their enzymatic significance. We will describe a cell-free preparation from peas that evolved ethylene in both an enzymatic and nonenzymatic manner.

Etiolated epicotyls of 8-day-old peas (Pisum sativum L. var. Alaska) grown on vermiculite at 23°C were harvested and stored in a freezer until used. All procedures were performed between 0° and 4°C. About 250 grams of epicotyls were chopped into small pieces, added to 125 ml of glass-distilled water, and ground in a Waring Blendor until a smooth paste was formed. The paste was squeezed through cheesecloth and the liquid centrifuged at 12,000g for 30 minutes to remove the larger particles.

The enzyme was prepared by adding 11 grams of ammonium sulfate to 50 ml of the crude supernatant, centrifuging at 10,000g for 15 minutes, and discarding the pellet. An additional eight grams of ammonium sulfate was added, the solution was centrifuged at 10,000g for 15 minutes, and the resultant pellet was taken up in ten ml of water to be dialyzed overnight against water. The dialyzed protein was then cleared by centrifugation at 10,000g for 15 minutes and found to have a protein concentration of about seven mg of protein per ml as determined by absorption at 260 and 280 mμ.[8] The enzyme prepared by this method appeared to be soluble because centrifugation at 144,000g for one hour did not result in a significant decrease in activity.

It was also possible to prepare an active protein fraction from the original crude supernatant by adding CM-Sephadex C-50 to remove the substances of lower molecular weight. However, the ammonium sulfate method was used for the experiments described here in order to concentrate the protein. The enzyme was stable at 0°C with a 50 per cent loss in activity after two days.

Substrate was prepared from the original crude supernatant by adding 1.5 volumes of acetone per volume of supernatant to precipitate the protein. The precipitate was removed by centrifugation at 10,000g for 15 minutes, and the acetone was evaporated off under vacuum at 30°C. This crude substrate was stable at 0°C and lost a negligible amount of activity over three days. It is also possible to prepare substrate by dialyzing the crude supernatant against water and evaporating the resultant diffusate down to the original volume of crude supernatant. Although identical results were obtained with both procedures, the acetone precipitation method was used as a matter of convenience.

Various mixtures of substrate and protein were prepared with 125 μmoles of acetate buffer (pH 4.6) and 0.125 μmole $Mn(NO_3)_2$ and placed in 5-ml syringes (liquid volume 2.5 ml, gas volume 2.5 ml) fitted with rubber vaccine caps so that a 2-ml gas sample could be withdrawn for analysis (Table I). The syringes, held at 29°C, were shaken 80 times a minute with an amplitude of two cm. Ethylene was determined by gas chromatography by a method described earlier.[9]

TABLE I. ETHYLENE EVOLUTION BY CELL-FREE PEA EXTRACTS[a]

Mg Protein	Ml Substrate	Ethylene, mμl/15 min
0.0	1.4	0.10
0.1	1.4	0.30
0.2	1.4	0.60
0.4	1.4	1.70
0.5	0.0	0.0
0.5	0.5	1.1
0.5	1.0	2.1
0.5	1.5	2.7
0.5	2.0	2.7

a. 125 μmoles acetate buffer (pH 4.6); 0.125 μmole $Mn(NO_3)_2$; 2.5 ml liquid volume; 29°C.

Data in Table I show that there was no ethylene evolution by the enzyme alone and a slight evolution of gas by the substrate alone. Stepwise increases in protein resulted in similar increases in ethylene evolution. Increasing the amount of substrate while holding the protein concentration constant resulted in an increasing rate of gas production until the process was substrate-saturated. Fifteen minutes were routinely used to determine the rate of ethylene evolution because gas production was linear within this time.

Heating at 100°C for ten minutes destroyed all enzymatic activity and heating at 60°C for ten minutes lowered the activity to half that of the original. The substrate was also found to be heat labile. Heating at 100°C for ten minutes destroyed all activity and 50 per cent of the activity was lost at 60°C. The substrate became inactive insofar as an

enzymatic release of ethylene was concerned if the pH was raised to 9 for ten minutes and then lowered to the original pH of 6. Half of its activity was destroyed at pH 7.5. The substrate was stable between pH 6 and 4, but lower pH's partially destroyed activity. For example, pH 3 for ten minutes caused a 25 per cent decrease in activity.

With 50 mM of acetate buffer the pH optimum for the reaction was between 4.5 and 4.7. A similar pH optimum was observed with citrate buffer, although the rate was one-fourth that in acetate.

A series of ions were tested for their effect on the reaction and only Mn^{+2} at 5×10^{-5} M concentration stimulated ethylene liberation. Greater concentrations of Mn^{+2} progressively inhibited the reaction. Other ions tested that had either no effect or inhibited in a concentration range of 10^{-2} to 10^{-4} M were Al^{+3}, Ca^{+2}, Co^{+2}, Cu^{+2}, Fe^{+2}, Fe^{+3}, K^{+}, Mg^{+2}, Mo^{+6}, Na^{+}, Ni^{+2}, and Zn^{+2}.

The coenzymes ATP, CoASH, thiamine pyrophosphate, NAD, NADH, NADP, FMN, and FAD, at a range of concentrations, either had no effect or inhibited the ethylene evolving reaction.

The most effective inhibitor tested was CN^{-}, which inhibited evolution of ethylene by 50 per cent at 10^{-4} molar concentration. Azide and NaF produced a 50 per cent inhibition at 10^{-3} M; Hg^{+2}, dinitrophenol, iodoacetate, and hydroxylamine inhibited 30 per cent or less at 10^{-2} M. EDTA at a concentration of 5×10^{-4} M resulted in a 50 per cent decrease in ethylene evolution. Addition of larger amounts of Mn^{+2} had only a slight effect in relieving this inhibition.

Compounds containing SH groups also inhibited the production of ethylene. Thioglycolic acid was most effective (100% at 10^{-3} M); thioglycerol, cysteine, reduced glutathione, and CoASH had progressively less effect. Ascorbic acid also inhibited ethylene production. In the presence of these compounds our preparations evolved ethane in quantities equivalent to the amounts of ethylene normally produced. All of the above compounds initiated ethane evolution from the substrate alone, but for cysteine, glutathione, thioglycerol, and ascorbate, the presence of protein enhanced the rate of ethane evolution.

Ethanol, ethionine, methionine, glycine, ethane, glycolic acid, glyoxylic acid, and acetyl-coenzyme A were added to the enzyme in the presence of a limiting amount of substrate to determine if they were possible precursors of ethylene. None had any stimulatory effect.

Although indoleacetic acid stimulated ethylene production in intact plants,[9] it was without effect on gas production from cell-free preparations.

In addition to the enzymatic production of ethylene from the substrate, it was found that Fe^{+2} ions and FMN (5×10^{-3} M) would cause ethylene evolution from the substrate alone at a rate greatly exceeding (sevenfold for FMN and fourfold for Fe^{+2}) that obtained with a saturating amount of enzyme. In addition, the total amount of ethylene evolved in the non-enzymatic reaction was greater than that in the protein-mediated one.

Thus it appears that ethylene evolution from cell-free preparations of etiolated peas can be mediated by both enzymatic and nonenzymatic means; this may explain in part some of the conflicting reports of earlier workers. It should be possible to determine the compound or compounds involved in the biosynthesis of ethylene for the system described in this paper.

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